Sensitivity of solid culture, broth culture, and real-time PCR assays for milk and colostrum samples from *Mycobacterium avium* ssp. *paratuberculosis*-infectious dairy cows

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ABSTRACT

Mycobacterium avium ssp. *paratuberculosis* (MAP) can be shed in feces, milk, and colostrum. The goal of this study was to assess assays that detect MAP in these sample types, including effects of lactation stage or season. Understanding the performance of these assays could improve how they are used, limiting the risk of infection to calves. Forty-six previously confirmed MAP-positive cows from 7 Atlantic Canadian dairy farms were identified for colostrum sampling and monthly sampling of milk and feces over a 12-mo period. Samples were assayed for MAP using solid culture, broth culture, and direct real-time PCR (qPCR). Across assay types, test sensitivity when applied to milk samples averaged 25% of that when applied to fecal samples. For colostrum samples, sensitivity depended on assay type, with sensitivity of qPCR being approximately 46% of that in feces. Across sample types, sensitivity of qPCR was higher than that of the other assays. Sensitivity of qPCR, when applied to milk samples, was significantly higher in summer than in other seasons. Summer was also the season with highest agreement between milk and fecal samples collected within the same month. Our results suggest that qPCR would detect more cows shedding MAP in their milk and colostrum than solid or broth culture assays, particularly during the summer, thus providing better management information to limit exposure of calves to this infectious organism.

Key words: milk and colostrum, real-time PCR, season, *Mycobacterium avium* subspecies *paratuberculosis*

INTRODUCTION

Johne's disease, or paratuberculosis, is caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and is

an incurable, production-limiting disease of dairy cattle. Although the disease manifests as chronic diarrhea in adult cows (affected cows), it has a characteristic long preclinical stage (infected cows), ranging from 2 to 10 yr, during which infected cows can intermittently shed detectable bacteria (infectious cows) (Whittington and Sergeant, 2001; Nielsen and Toft, 2008). In addition to fecal shedding, MAP can also be shed via the mammary gland into milk and colostrum (Lombard, 2011). Calves are the most susceptible to infection, with the highest risk occurring in calves less than 1 mo of age (Sweeney, 2011). Calves that have not become infected in utero are most likely to become infected after birth by ingesting MAP through either directly infected or fecal-contaminated colostrum or milk, or by ingesting MAP-contaminated feces from the udder or environment (Sweeney, 2011). It has been reported that affected cows can shed from <100 to 1,000 cfu of MAP per milliliter of milk (Sweeney et al., 1992; Giese and Ahrens, 2000). A calf risks infection by ingesting as little as 50 cfu of MAP (Chiodini, 1996). In a study by Streeter et al. (1995), 3 times as many preclinical cows shedding MAP in their feces had the bacterium isolated from colostrum as from milk, and approximately 22% of preclinical cows were found to be shedding MAP in colostrum. The sensitivity with which MAP is detected varies with infection stage, assay type, and laboratory method. A recent meta-analysis of MAP shedding through milk, as detected by culture and PCR, found an overall apparent MAP prevalence of 20% in individual milk samples (Okura et al., 2012). Stressful situations, including parturition, peak milk production, and environmental changes, can generate or increase clinical signs and subsequent MAP shedding (Sweeney, 2011; Bradner et al., 2013a). For MAP, the direct and chronic effect of stress-hormones on the macrophage-pathogen interplay can negatively affect disease resistance in the host and enhance disease progression and bacterial shedding (Verbrugghe et al., 2012).

Culture is currently the reference standard for direct bacterial identification (Bölske and Herthnek, 2010).

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Unfortunately, the slow growth rate of MAP requires culture incubation times of up to 7 wk on broth medium and 16 wk on solid medium (Whittington, 2010). As MAP bacterial numbers inherently tend to be much lower in milk and colostrum than in feces, additional sedimentation, centrifugation, and filtration steps can improve the bacterial concentration. However, subsequent decontamination with incubation in an antimicrobial brew can decrease the bacterial load in the sample. In an effort to minimize this bacterial loss, it is now generally accepted that both the cream and pellet portions should be incorporated in the pre-culture or DNA extraction steps following centrifugation (Gao et al., 2005, 2009; Bradner et al., 2013a).

Molecular testing via real-time PCR methods [quantitative (q)PCR] has been developed for MAP detection directly or as culture confirmation, being time and cost efficient compared with culture methods. For example, in Atlantic Canada, an individual qPCR assay costs Can\$25 compared with Can\$40 per culture assay (www.atlanticjohnes.ca/theprogram.php#cowtesting; accessed June 8, 2015). Although culture and qPCR are commonly used and readily available pathogen detection assays, qPCR detects both viable and nonviable MAP, and takes less than 48 h to obtain results. In addition, no decontamination techniques are required before DNA extraction (Bölske and Herthnek, 2010). Although the MAP-specific insertion element *IS900* is the usual sequence targeted, other gene elements detected in milk and colostrum include *F57* and *IS-MAP02* (Pithua et al., 2011a; Hanifian et al., 2013). Another sequence unique to MAP is *hspX* (Ellingson et al., 1998; Bölske and Herthnek, 2010), as used in the Tetracore real-time qPCR kit for MAP detection (Tetracore, Rockville, MD).

The incurable nature and insidious preclinical phase of paratuberculosis challenge the identification of infected and infectious animals. Identification is essential to decreasing transmission into and within the herd and, most importantly, decreasing exposure to calves (Whitlock, 2010). Control of fecal contamination of the environment, particularly areas specified for parturient and dry cows, and segregation of high-shedding cows becomes a priority (Pithua et al., 2013). Another priority involves feeding calves colostrum or waste milk that does not contain the organism (Diéguez et al., 2008; Pithua et al., 2011b). It is often stated that pooled colostrum and colostrum or waste milk from MAP-infected cows should not be used. Rather, milk replacer, properly pasteurized waste milk, or milk from a single noninfected dam is recommended (Nielsen et al., 2008; Garry, 2011; Lombard, 2011). Despite being widely endorsed, there is conflicting evidence about the efficacy of these recommendations. In a recent study,

MAP exposure via colostrum had no effect on subsequent MAP infection, although the authors advised that these results be cautiously interpreted (Pithua et al., 2011a). Another study found that in an infected herd, more than 81% of the MAP-positive colostrum or MAP-positive teat swabs came from potential environmental seeding of MAP rather than direct shedding (Pithua et al., 2011b).

Mycobacterium avium ssp. *paratuberculosis* has been isolated from patients with Crohn's disease, spurring public health concerns of a zoonotic link between MAP and Crohn's disease, a chronic enteritis in humans. Indeed, MAP in milk and milk products for human consumption is a potential human health concern, particularly as research has shown that, to a certain extent, MAP can survive commercial HTST pasteurization techniques for milk (Grant et al., 2002; Manning and Collins, 2010; Van Brandt et al., 2011). Recently, MAP DNA has been found in some commercial baby formulas, using both *IS900* and *F57* qPCR (Hruska et al., 2005, 2011).

For producers, the ability of MAP to survive some pasteurization techniques presents a further concern. Some producers rely on on-farm pasteurized colostrum and milk for their calves. Although most MAP can be killed in colostrum at 60°C for 60 min (a recommended protocol for on-farm pasteurization), the results are still variable and depend on the MAP load within the sample (Godden et al., 2006).

Seasonal effects on MAP detection in pasteurized milk samples for retail sales have been reported (Ellingson et al., 2005). A British study found higher levels of MAP in retail milk between December and March (Millar et al., 1996; Grant et al., 2002), but limited information is available regarding effects of stage of lactation and season on MAP detection in milk and colostrum samples. Factors affecting MAP concentration in bulk tank milk include seasonal calving and stage of Johne's disease (Cazer et al., 2013). As well, Bradner et al. (2013b) and Stabel et al. (2014) detected higher MAP shedding in colostrum and milk in more advanced disease stages and in early-lactation periods.

Understanding patterns of MAP shedding, not just in feces but also in milk and colostrum, can support development of more time-efficient and cost-efficient detection strategies. The first objective of our study was to assess the sensitivity of 3 MAP pathogen detection assays (solid culture, broth culture, and qPCR) when each was applied to milk samples and colostrum samples. To do so, sensitivity in milk and colostrum was assessed across assay-specific shedding categories; and, using samples from previously determined MAP-infectious cows, the sensitivity of the assays in milk and colostrum was compared with the sensitivity in feces. A

second objective was to identify any effects of season or lactation stage on MAP detection within milk and colostrum. This knowledge is important for improving the use of diagnostic assays for MAP infection in milk and colostrum. Improved recommendations for control and monitoring of this disease within a herd can thereby assist in minimizing the risk of exposure to calves.

MATERIALS AND METHODS

Farm and Cow Selection

From a companion project assessing MAP herd diagnostics in Atlantic Canadian dairy herds (Lavers et al., 2013), we purposively selected 7 dairy farms: 3 from Prince Edward Island and 4 from New Brunswick, Canada. Within-herd MAP prevalence (as determined by pooled fecal culture, followed by individual fecal culture of positive pools and confirmed with acid-fast staining and qPCR, 3 times over 12 mo) in these MAP-positive herds ranged from 3 to 15% in the previous year (C. Lavers, Atlantic Veterinary College, Charlottetown, PE, Canada; personal communication); and herd size ranged from 83 to 490 cows per herd.

From these herds, 46 MAP-infectious Holstein cows were recruited. Inclusion criterion for recruitment of these 2 to 15 cows per farm was active shedding of detectable amounts of MAP in their feces (target condition), as determined from the companion project (Lavers et al., 2013) as described above. A cow was labeled MAP-infectious if it was fecal culture positive (confirmed with acid-fast stain and qPCR) at least once during a 1-yr period before the start of the current study (case definition). Although this selection process may have also included intermittently shedding MAP-infected cows (Nielsen and Toft, 2008), we followed the guidelines of Gardner et al. (2011) and Nielsen and Toft (2008), identifying the enrolled MAP-shedding cows as MAP-infectious for this study. At each sampling period, age, parity, and DIM were also recorded for each cow.

Sample Collection

Protocols were approved by the Animal Care Committee at the University of Prince Edward Island before the study. Fecal samples from each cow were collected monthly with clean, individual rectal sleeves, without lubrication, for up to 12 mo (from July 2010 to December 2011), as long as the cow remained in the herd. From the 46 MAP-infectious cows, 36 cows had colostrum samples collected by the farmer within 24 h of parturition. Clean milk samples were collected monthly either by the project personnel or by the farmers (these samples were subsequently stored on-farm at -20°C),

and all samples were transported, monthly, on ice to the Maritime Quality Milk Laboratory at the University of Prince Edward Island, Canada, where they were frozen at -80°C until processing. The Maritime Quality Milk Laboratory is USDA proficiency tested for fecal culture and qPCR.

Laboratory Procedures

Fecal Solid Culture. Solid cultures were performed on Herrold's egg-yolk medium (HEYM) slants supplemented with mycobactin J and amphotericin B, naladixic acid, and vancomycin (Becton, Dickinson, and Company, Sparks, MD). Procedures followed, in part, methods described by Stabel (1997). Briefly, 3 g of thawed fecal sample was added to 30 mL of half-strength brain-heart infusion (BHI) with 0.9% hexadecylpyridinium chloride monohydrate (HPC; Sigma Chemical Company, St. Louis, MO). After sitting at room temperature for 30 min, 15 mL of supernatant was transferred to a new 50-mL polypropylene tube and centrifuged at $1,700 \times g$ for 20 min at room temperature. The supernatant was then discarded. To the remaining pellet, 30 mL of the 0.9% HPC-BHI solution was added, and the suspension was incubated overnight at 37°C . The next day, the suspension was centrifuged at $1,700 \times g$ (IEC CL31 Multispeed Centrifuge, Thermo Scientific, Oakwood Village, OH) for 20 min at room temperature, and the supernatant was subsequently discarded. To the remaining pellet, 100 μL of 0.9% saline solution was added. Then, 100 μL of the resuspended sample was inoculated onto the HEYM slant, which was placed at a slight incline at 37°C . After the inoculation dried, the cap was secured tightly and the tube placed vertically at 37°C for 84 d. Cultures were examined weekly with the aid of a dissecting microscope, and colony-forming units were recorded until 84 d of growth, until >100 cfu/tube was observed (labeled as >100 cfu), or until growth of competitive bacteria and fungi covered any detectable MAP colonies. If overgrowth of competitive organisms occurred, the sample was labeled as contaminated. All culture-positive results were confirmed with acid-fast stain and qPCR as described below.

Milk and Colostrum Solid Culture. Procedures followed methods described by Donaghy et al. (2008) for milk and by Godden et al. (2006) for colostrum. Because culture decontamination techniques can decrease MAP concentration, and MAP concentration was expected to be lower in milk and colostrum than in feces, we followed the protocol described below, which includes modifications as a result of consultation with L. Mutharia (Ontario Veterinary College, Guelph, ON, Canada; personal communication), and J. DeBuck (University of Calgary, AB, Canada; personal commu-

nication). In brief, 30 mL of milk sample or colostrum sample was centrifuged at $2,800 \times g$ for 30 min at room temperature (21°C) and the whey fraction discarded. The cream and pellet fractions were resuspended completely in 30 mL of 0.75% HPC. Following incubation at room temperature for 5 h and centrifugation at $2,800 \times g$ for 30 min at room temperature, the liquid phase was discarded, and the pellet was diluted in 100 μ L of a 0.9% saline solution and vortexed to resuspend the pellet. From this sample, 100 μ L was inoculated onto the HEYM slant and placed at a slight incline at 37°C until dry, after which the cap was secured tightly and the tube placed vertically at 37°C for 84 d. Weekly examinations were performed with the aid of a dissecting microscope and counts (cfu) were recorded until 84 d of growth, until >100 cfu/tube was observed (labeled as >100 cfu), or until growth of competitive organisms covered any detectable MAP colonies. If overgrowth of competitive organisms occurred, the sample was labeled as contaminated. All culture-positive results were confirmed with acid-fast stain and qPCR, as described below.

Fecal Broth Culture. The ESP culture system II (Trek Diagnostic Systems, Thermo Scientific) was used to obtain quasi-quantitative days-to-positive counts. In this system, MAP growth is signaled by decreased pressure detected in the headspace of the medium bottle. As per the kit's instructions, 2 g of thawed fecal sample in 35 mL of sterile water was shaken for 10 min on an automatic shaker (Mistrel Multi-Mixer 4600, Barnstead Lab-Line, Melrose Park, IL) and then allowed to settle for 30 min at room temperature. Five milliliters from the top one-third of the solution was then added to 25 mL of 0.9% HPC-BHI solution. This sample was incubated overnight at 36°C, and then centrifuged at $1,500 \times g$ for 20 min. One milliliter of an antibiotic brew (18.5 mg/mL BHI, 975 μ L/mL deionized water, 100 μ g/mL vancomycin, 100 μ g/mL nalidixic acid, and 50 μ g/mL amphotericin B) was added to the pellet and resuspended by vortexing, and the mixture was then incubated overnight at 36°C. The following day, 2.5 mL of a solution comprising 1 mL of ESP Growth Supplement, 1 mL of Egg Yolk Supplement, and 0.5 mL of Antibiotic Supplement (all reagents from the Para-JEM kit) was added to a Para-JEM broth mycobottle (Thermo Scientific, Nepean, ON, Canada), followed by the addition of 1 mL of the decontaminated sample. The culture was then incubated in the Trek incubator until growth was signaled, up to a maximum of 49 d or until a positive pressure signal was detected. All samples incubated to 49 d and all positive samples were confirmed with acid-fast stain. Any positive results on either broth culture or acid-fast stain were confirmed with qPCR as described below.

Milk and Colostrum Broth Culture. Briefly, 30 mL of the milk or colostrum sample was centrifuged at $2,800 \times g$ for 30 min. The whey was decanted, and 30 mL of 0.75% HPC was added to the remaining cream and pellet. The suspension was then incubated for 4 h at room temperature. Afterward, the sample was centrifuged at $1,500 \times g$ for 20 min, and the remaining procedure for fecal broth culture was followed as described above.

Fecal Direct qPCR. Methods followed the procedures listed for the VetAlert Johnes's Real-Time PCR kit (Tetracore), targeting the *hspX* gene. Briefly, 2 g of thawed fecal sample was vortexed in 35 mL of DNase-free water and shaken for 15 min on an automatic shaker. The sample was then incubated for 30 min at room temperature. The top 20 mL of the solution was centrifuged at $2,500 \times g$ for 10 min at room temperature. One milliliter of $1\times$ Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) was used to resuspend the pellet.

Extraction of DNA was performed as follows: 1 mL of the solution was added to a cell-disruption tube containing sterile glass beads and bead-beat (Mini-Beater 8, BioSpec Products, Bartlesville, OK) at 4,800 oscillations/min for 5 min, and then centrifuged for 10 min at $16,000 \times g$ (IEC MicroCL 21R Microcentrifuge, Thermo Scientific). The decanted supernatant was combined with 100 μ L of Nucleic Acid buffer (NAB, Tetracore) by vortexing, and the solution was then centrifuged at $1,200 \times g$ for 3 min. The liquid fraction was discarded, and the remaining pellet was resuspended in 560 μ L of Binding buffer (Tetracore) and incubated for 10 min at room temperature. Then, 560 μ L of 100% ethanol was added, after which 630 μ L of the sample was placed in a spin column and centrifuged at $5,200 \times g$ for 1 min. The remaining sample was added to the spin column and centrifuged at $5,200 \times g$ for 1 min. Next, 500 μ L of Wash buffer A (Tetracore) was added to the spin column, which was centrifuged at $5,200 \times g$ for 1 min, followed by the addition of 500 μ L of Wash buffer B (Tetracore) to the spin column and centrifugation at $12,000 \times g$ for 3 min and again at $16,000 \times g$ for 1 min without additional buffers. The spin column was placed into a microcentrifuge tube, 50 μ L of deionized water was added, followed by a 1-min incubation at room temperature, and then centrifugation at $5,200 \times g$ for 1 min. The DNA elution was saved at 4°C for up to 48 h or at -20°C for longer storage.

The qPCR procedure was a fluorogenic probe hydrolysis assay, in which 22.5 μ L of thawed master mix (Tetracore) and 2.5 μ L of the eluted DNA was added to a thermocycler reaction tube. For quality control, positive (Tetracore) and negative (DNA-grade water, Fisher Scientific Co., Ottawa, ON, Canada) control samples

were included in each run of qPCR. The reaction tubes were centrifuged for approximately 15 s in a Cepheid microcentrifuge before being placed in the Cepheid SmartCycler II Thermocycler (Cepheid, Sunnyvale, CA). After an enzyme activation step (95°C), a 2-step cycling reaction (95°C and 62°C) was used. The cut-off value for the positive control was set to between 20 and 26 cycle threshold values. Samples that were positive within 42 cycles were considered MAP positive.

Milk and Colostrum Direct qPCR. Milk and colostrum direct qPCR procedures followed a modified version of the method described by Gao et al. (2007). For milk, 30 mL of sample was heated in a water bath (95°C) for 10 min and then cooled in ice water for 10 min. The milk sample was then centrifuged at $2,800 \times g$ for 30 min at room temperature. The pellet and cream were resuspended in 6 mL of 0.75% HPC and incubated for 30 min at room temperature. The sample was then centrifuged at $2,000 \times g$ for 15 min at room temperature. The liquid phase and cream were decanted. The pellet was transferred to a 2-mL microcentrifuge tube, and 1 mL of Tris (pH 8) and 20 μ L of protein kinase were added. The sample was transferred to a cell-disruption tube containing sterile glass beads and homogenized in the bead beater (Mini-Beater 8, BioSpec Products) at 4,800 oscillations/min for 3 min, incubated in a water bath for 10 min at 56°C, and then homogenized again in the bead beater as before, and cooled on ice. The steps to complete the extraction and qPCR reaction were performed as described above for fecal direct qPCR. For quality control, positive (Tetracore) and negative (DNA-grade water, Fisher Scientific Co.) control samples were included in each run of qPCR. To limit the risk of false-negative samples, an inhibition control (Tetracore) was also added.

For colostrum, 30 mL of sample was centrifuged at $2,800 \times g$ for 30 min at room temperature, and the whey and cream decanted. The pellet was transferred to a 2-mL microcentrifuge tube, and 1 mL of Tris (pH 8) was added. The sample was transferred to a cell-disruption tube containing sterile glass beads and homogenized in the bead beater (Mini-Beater 8, BioSpec Products) at 4,800 oscillations/min for 5 min. The steps to complete the extraction and qPCR reaction were performed as described above for fecal direct qPCR. For quality control, positive (Tetracore) and negative (DNA-grade water, Fisher Scientific Co.) control samples were also included in each run of qPCR. To limit the risk of false-negative samples, an inhibition control (Tetracore) was also added.

Culture Confirmation. All broth and solid culture results were confirmed with acid-fast stain, and any positive results on either culture or acid-fast stain

were further confirmed with qPCR. For broth culture confirmation, the bottle was placed in an automatic shaker for 5 min. One milliliter of the broth culture was added to a cell-disruption tube. The procedure then followed the DNA extraction and qPCR methods described above. For solid culture confirmation, 2 or 3 colonies were collected from the medium with a sterile loop and mixed with 1 mL of sterile 0.9% saline in a sterile centrifuge tube. One milliliter of the solution was then placed in a cell-disruption tube, and again the DNA extraction and qPCR test procedures, as described above, were followed.

Statistical Analysis

Statistical analysis of results was performed using STATA/IC 12 (StataCorp LP, College Station, TX), and statistical significance was set at a *P*-value of < 0.05. Sensitivity (proportion of positive results in previously determined MAP-infectious cows) was recorded along with a 95% confidence interval for each assay for all sample types over assay-specific shedding levels. For solid culture, low shedding was established as <10 cfu per culture tube, moderate shedding from 10 to 50 cfu per tube, and high shedding at >50 cfu per tube (Crossley et al., 2005). For broth culture, high shedding was described as <21 d to positive, moderate shedding between 21 and 28 d to positive, and low shedding between 29 and 49 d to positive (Shin et al., 2000, 2001). Proportions of detectable MAP shedding in milk when there was concurrent fecal shedding per month were further assessed.

Population-averaged, cow-level, generalized estimating equation models were used for the milk analysis. This model structure best handled the repeated samples per cow and, given the data structure and exploration of various correlation structures, an autoregressive (AR1) within-cow correlation and robust standard errors were selected as most appropriate. Separate models were run for each of the dichotomous outcomes of solid culture, broth culture, and qPCR. Season (categorical) and lactation stage (categorical) were the main predictors of interest. Other predictors included farm (categorical) and age (categorical; yr) to assess for confounding. Seasons were categorized as follows: summer = July through September, fall = October through December, winter = January through March, and spring = April through June. Lactation stages were divided as follows: <60, 60 to 99, 100 to 239, and ≥ 240 DIM.

Because there were no repeated colostrum samples, colostrum results were analyzed with simple logistic models (one for each of the 3 dichotomous outcomes of solid culture, broth culture, and qPCR), including

Figure 1. Sensitivity (%), with 95% confidence intervals, of solid culture (Herrold's egg yolk medium with mycobactin J), broth culture (Trek ESP system; Trek Diagnostic Systems/Thermo Scientific, Oakwood Village, OH), and real-time PCR (qPCR; VetAlert, Tetracore, Rockville, MD) for fecal, milk, and colostrum samples collected monthly for a period of 12 mo from 46 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds. Within each bar, the number of observations per category is shown.

the main categorical predictor of interest for season and assessing any other potential categorical predictors for farm, age, or parity. Any predictors with P -values < 0.2 in univariable models were further analyzed in multivariable models.

RESULTS

Descriptive Data

Cows ranged in age from 2 to 8 yr (median = 4 yr) and from first to seventh parity (median = 2). Because data were sparse above 400 DIM, and to stay within a typical lactation length, analyses were limited to results below this threshold, resulting in 298 fecal samples and 304 milk samples for analysis. Thirty-seven colostrum samples were collected from unique parturitions from 36 cows. On 296 occasions, milk and fecal samples were collected within the same month, and on 37 occasions, colostrum and fecal samples were collected within the same month.

For the fecal samples, 18 of 298 solid cultures were contaminated with overgrown fungal organisms (6.0% contamination rate), whereas for milk samples, 13 of 304 solid cultures were contaminated (4.3% contamination rate). Contaminated solid cultures were removed from the analyses. In addition to these samples, 1 fecal sample for qPCR analysis was lost, and 31 milk samples and 6 colostrum samples had insufficient volume for analyzing with all 3 assays.

We detected fecal shedding (with any of the 3 organism detection methods) in 45 of the 46 cows in our

case group, with 31 of these cows shedding detectable amounts of MAP (by one or more methods) in their feces at 100% of their sampling times, and the remaining 14 cows shedding at between 25 and 88% of their sampling times. Furthermore, of the same 46 cows in our case group, 10 cows never shed detectable MAP in their milk during the study period.

Sensitivity of Assays. Using a history of MAP fecal shedding in the previous year to define the reference infection status, sensitivity values for each of the 3 assays were determined for each of the 3 sample types (Figure 1). Predicted 95% CI were included when more than 40 observations per assay were available. Sensitivity was higher in fecal samples compared with milk and colostrum samples. For all sample types, qPCR had the highest sensitivity, followed by broth culture and then solid culture.

The level of shedding measured by solid culture (Crossley et al., 2005) and by broth culture (Shin et al., 2000, 2001) for each sample type is presented in Table 1. Using broth cultures, no high-shedding cows were identified by both colostrum and milk sampling. Using solid cultures, high-shedding cows were identified with both milk and colostrum samples. However, comparisons of solid and broth culture outcomes should be interpreted cautiously, because the assay-specific shedding classifications may not be equivalent.

Concurrent Milk and Fecal Detection Patterns

Figure 2 shows detection of MAP in both milk and feces collected from the same cow during the same

Table 1. Percentage of solid culture (Herrold's egg yolk medium with mycobactin J) and broth culture (Trek ESP system; Trek Diagnostic Systems/Thermo Scientific, Oakwood Village, OH) outcomes across shedding levels for fecal, milk, and colostrum samples collected monthly for a period of 12 mo from 46 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds¹

Culture	Sample	Negative	Low shedding	Moderate shedding	High shedding
Solid culture ²	Feces	53.3 (45.3–59.3) [144]	15.9 (11.5–20.3) [43]	8.1 [22]	22.6 (17.6–27.6) [61]
	Milk	86.6 (82.7–90.5) [252]	9.3 [27]	0.7 [2]	3.4 [10]
	Colostrum	97.0 [32]	0	0	3.0 [1]
Broth culture ³	Feces	45.0 (39.3–50.6) [134]	30.9 (25.6–36.1) [92]	9.1 [27]	15.1 (11.0–19.2) [45]
	Milk	85.9 (81.6–90.1) [225]	11.8 [31]	2.3 [6]	0
	Colostrum	89.3 [25]	10.7 [3]	0	0

¹Values are percentages (with 95% CI in parentheses if >40 observations available); number of observations at the corresponding shedding level is shown in square brackets. Rows may not sum to 100.0 due to rounding.

²Solid culture: low (<10 cfu per culture tube), moderate (10 to 50 cfu), and high (>50 cfu) (Crossley et al., 2005).

³Broth culture: low (>28 d to a positive signal), moderate (21 to 28 d), high (<21 d) (Shin et al., 2000, 2001).

month. When MAP was detected in feces with any of the 3 assays, 36.5% of these cows were also found to be shedding MAP in their milk, with at least 1 of the 3 assays used. In milk samples, qPCR identified an average of 22.7% of those MAP-positive cows concurrently

shedding MAP in feces. Furthermore, from the previously determined MAP-infectious cows, 6.4% (95% CI: 2.3 to 10.4%) of the negative fecal solid culture results and 9.2% (95% CI: 4.0 to 14.4%) of the negative fecal broth culture results obtained during this study were

Figure 2. Percentage of positive milk sample results (with 95% confidence intervals) obtained from solid culture (Herrold's egg yolk medium with mycobactin J), broth culture (Trek ESP system; Trek Diagnostic Systems/Thermo Scientific, Oakwood Village, OH), and real-time PCR (qPCR; VetAlert, Tetracore, Rockville, MD) when there was a concurrently positive fecal result, detected with one of the same 3 assay types, for samples collected over 12 mo from 46 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds. Milk result was classified as positive if there was a positive result on broth culture, solid culture, or qPCR assay. Within each bar, the total number of positive fecal results is shown; fecal result was classified as positive if there was a positive result on any one or more of broth culture, solid culture, or qPCR categories.

related to concurrent low shedding in milk, using the same culture method.

Seasonal and Lactational Patterns in Test Sensitivity

Colostrum results showed no significant association with season with any assay. We found no association between lactation stages and detection of MAP in milk with any assay. Direct qPCR results for milk samples showed a significant association with season ($P < 0.005$), with farm included as a potential confounding variable. Probabilities for positive qPCR results within seasons are shown in Figure 3. Contrasts confirmed a significant difference between summer and fall ($P < 0.005$) and between summer and winter ($P = 0.02$). Contrasts were borderline nonsignificant between summer and spring ($P = 0.07$) and between winter and fall ($P = 0.06$). The probability of obtaining a positive qPCR result in milk samples during the fall, winter, and spring seasons was only 43.7% (95% CI: 29.8 to 51.3%) of the probability of the test being positive in summer.

For culture of MAP in milk samples on solid medium, there was a noticeable pattern of increased failure of the decontamination procedures to eliminate competitive organisms on the medium (13 contaminated samples), leading to more samples being classified as contaminated in the summer (6 samples from 65 total samples in the summer) and fall (5 from 77 samples) than in the winter (1 from 45 samples) or spring (1 from 57 samples).

Figure 3. Predicted probability (%) with 95% confidence intervals for positive (+) direct real-time PCR (qPCR; VetAlert, Tetracore, Rockville, MD) across seasons for milk samples collected monthly over a period of 12 mo from 46 *Mycobacterium avium* ssp. *paratuberculosis* infectious cows from 7 herds.

As season was a significant predictor of MAP shedding in milk, the least amount of MAP shedding in milk was detected during fall, when there was concurrent detectable fecal shedding. In contrast, the best agreement between milk and fecal shedding was seen during the summer season.

DISCUSSION

Johne's disease is one of the animal health priorities in the Canadian dairy industry. The disease has worldwide distribution (Collins, 2003; Singh et al., 2013) and was listed as having serious economic and zoonotic implications by the Office International des Epizooties in 2004 (OIE, 2004). The greatest animal health concern of MAP-infected milk and colostrum is transmission of the bacteria to calves, which are at the highest risk for infection (Sweeney, 2011). There is also a public health concern related to MAP in milk for human consumption (Grant et al., 2002; Manning and Collins, 2010; Van Brandt et al., 2011). These concerns have driven more research into efficient and effective methods of detection of the bacteria in milk and colostrum.

Sensitivity of Assays

Our study of comparing 3 assays to identify MAP was done in 2 ways. First, we looked at the sensitivity of each assay to identify our target condition of MAP-infectious cows. Second, milk, colostrum, and feces were compared as sample types for the assays.

This study found that regardless of the assay chosen, when the same assay was used for MAP detection in both milk and fecal samples, assays for milk were, on average, only 25% as sensitive as those for feces. In contrast, sensitivity within colostrum was dependent on which assay was used. Sensitivity of broth culture within colostrum was approximately 19% of that within feces, compared with 6% using solid culture and 46% using qPCR. It should be noted that sample size in the colostrum data was small, and results should be interpreted with caution.

Overall, for all 3 sample types, qPCR identified the greatest proportion of MAP-infectious cows within our study group. It is possible that low numbers of bacteria in the milk or colostrum samples or loss of viable cells via decontamination techniques led to no growth on culture media and, thereby, false negatives for low-shedding animals that could still be detected by qPCR. Culture of MAP in milk and colostrum can be difficult due to chemical inhibitors in the sample, the presence of low numbers of bacterial cells within the sample, or the clumping of bacterial cells (Gao et al.,

2005; Pinedo et al., 2008). Detection by culture can also be hindered via loss of bacterial cells either through centrifugation, where cells may be fractionated into the whey portion, or through decontamination, where MAP bacteria may be killed (Gao et al., 2005; Pinedo et al., 2008). Therefore, our study supports the use of a standardized qPCR methodology in Johne's control programs to identify low-shedding animals.

Accurate comparison of results among Johne's disease studies can be hindered by a lack of standardized methodology and consistent methods, such as those recommended by Gardner et al. (2011). Variations in methodology can be observed in culture techniques, media, PCR methods, and gene selection. In addition, the disease stage of cows within study groups could affect the degree of bacterial shedding into milk or colostrum. For example, in recent studies in which milk samples from clinical cows were assessed using a different liquid culture and decontamination protocol, MAP detection was approximately 9% with solid (HEYM) culture, 11% with broth (Bactec 12B) culture, and 39% with PCR (using *IS900* gene target) (Bradner et al., 2012, 2013b). These culture detection values were substantially lower than those from the current study. In contrast, a study by Gao et al. (2009) of 146 cows from 14 MAP-positive herds in southwestern Ontario compared solid culture and direct and nested PCR (using *IS900* gene target) in milk with solid fecal culture and detected more positive samples than we did in our study. From cows that previously tested positive by fecal culture, milk ELISA, or serum ELISA, they reported a sensitivity of 41.8% with fecal culture, 34.6% with milk culture, 28.4% with milk PCR, and 53.7% with milk nested PCR. In contrast, a study by Slana et al. (2008) found a sensitivity of 35% with solid culture of milk and a maximum sensitivity of 77.8% with milk qPCR. A recent study of Iranian farms found qPCR (using the *F57* gene) to be 10 times more sensitive than culture (using Middlebrook 7H11 medium) in detecting MAP in milk samples (Hanifian et al., 2013). Furthermore, previous studies have found that infected cows may shed between 2 to 9 cfu of MAP per 50 mL of milk (Sweeney et al., 1992; Rademaker et al., 2007), and that affected clinical cows may shed up to 1,000 cfu/mL of milk (Giese and Ahrens, 2000). Our sensitivity estimates were within the range of other studies, possibly resulting from our choice of target condition and reference standard. As noted above with the various examples, although in general PCR tends to detect more MAP-positive cows than culture methods, the trend is not concrete and the range of values varies greatly across the different assays, techniques, and protocols employed by the different studies.

Bradner et al. (2013b) stressed that for an infected cow, the level of MAP shed into milk and colostrum would increase as disease worsens, but Sweeney (2011) noted that MAP shedding could also increase with other disease conditions or stressors, such as parturition, that negatively affect the animal's immune status. Because MAP is an intracellular bacterium able to reside within macrophages and resist phagocytosis, a higher burden in colostrum would be expected during these events (Streeter et al., 1995). In our study, the sensitivity of culture methods applied to colostrum was very low (<10%). In contrast, the higher sensitivity of qPCR (approximately 35%) in our study may warrant its practical application, as Pithua et al. (2011b) found that not using colostrum from infectious cows could lead to an 18.2% lower risk of calves being exposed to MAP through colostrum. Additional research with larger sample sizes and, perhaps, comparisons of various techniques for detection of MAP in colostrum is recommended, as colostrum may well be a critical route of MAP transmission to calves. Young calves are at increased risk of MAP exposure through colostrum due to the greater likelihood of ingesting MAP-infected macrophages among the abundance of immunoglobulins present in colostrum (Streeter et al., 1995). In calves less than 1 d old, these colostral immunoglobulins are more readily absorbed ("open gut" syndrome) across the numerous mucosal lymphoid tissues present within the calves' small intestine and ileum (Whitlock and Buerge, 1996). Studies have shown that the higher the exposure rate and concentration, as well as the earlier the exposure occurrence, the less the exposed calf's immune system can resist subsequent infection, progression, and ultimately severity of the disease (Sweeney, 1996; Begg and Whittington, 2008; Mortier et al., 2013).

Our case definition was based on cows known to have shed MAP in their feces at least once within a 1-yr period before the start of this study. This criterion may have biased our results slightly as it is likely that some of these cows were shedding only intermittently during our study or perhaps even passively shedding (Nielsen and Toft, 2008). As the majority of cows were shedding detectable amounts of MAP during the study period, our case definition reflects the natural disease process, following the disease stage criteria of Nielsen and Toft (2008) and Gardner et al. (2011).

Furthermore, no standard shedding categories for milk and colostrum exist in the literature. It is possible that, because of the difficulty in culturing MAP in these samples compared with fecal samples and their inherently lower bacterial concentration, the shedding

categories we chose were too broad. However, we used the same shedding category cut-points for milk and colostrum samples as for fecal samples to compare counts (cfu) and days-to-positive counts with some measure of similarity.

Concurrent Milk and Fecal Detection Patterns

Our study found that when milk and feces were sampled concurrently, approximately one-third of cows with MAP-positive feces also had MAP-positive milk, regardless of the assay used. Again, literature results vary regarding milk and fecal agreement (Nielsen and Toft, 2008; Pinedo et al., 2008; Gao et al., 2009), as techniques and methods differ between studies. A recent study by Khol et al. (2013) in a low-prevalence herd (9%, as determined by fecal qPCR) used ELISA assays for blood samples, qPCR assays for fecal samples, as well as ELISA and qPCR assays for individual and bulk tank milk samples. They reported a significant correlation between MAP shedding in milk and feces, yet warned that shedding into milk may only occur for a limited time and may often be too low for detection, even with PCR. It has been suggested that increasing MAP levels in milk should correspond with increased fecal shedding and, ultimately, the stage of disease (Sweeney et al., 1992; Streeter et al., 1995). In our data, few cows had high levels of MAP in their milk. Gao et al. (2009) suggested that, because shedding in milk and feces may not coincide, both of these sample types should be cultured to identify as many infectious (including low-shedding) cows as possible. The cost effectiveness of such an approach would need to be investigated. In our data, approximately 10% of low MAP shedding in milk, detected by broth culture, was observed when concurrent fecal broth cultures were negative. Therefore, colostrum, milk, or waste milk from fecal test-negative cows in herds with Johne's disease could still be a source of MAP for calves. In these cases, it may be warranted to include milk testing (particularly with qPCR) as part of the herd's control program.

Seasonal and Lactational Patterns in Test Sensitivity

In our study of infectious cows, we found no significant associations between lactation stage and MAP shedding in milk with any of the assays. However, Bradner et al. (2013b) reported increased MAP detection in milk at the beginning of lactation (0 to 60 DIM) in affected Johne's diseased cows, and Stabel et al. (2014) reported the same for subclinical and affected cows. For milk samples, sensitivity qPCR was progres-

sively higher than that of both culture methods after 100 DIM and particularly after 240 DIM. Days in milk may have reflected progression of the disease within our study population.

More discordancy between concurrent MAP-shedding in feces and milk was observed during the fall months. In contrast, the greatest agreement was seen in the summer months, which corresponded with a relative improvement in sensitivity of qPCR in milk samples over fecal samples (Laurin et al., 2015). Our results of higher sensitivity in milk during summer (July through September) contradict results of a study from England and Wales by Millar et al. (1996), which found that seasonal patterns in MAP detection in retail milk peaked in autumn and winter. Seasonal patterns may depend on geographic location, but results could be extrapolated among locations with similar weather patterns. Crossley et al. (2005) also stress that milk policy and financial reasons involving milk supply and demand can determine whether MAP-infectious cows are kept on farm during certain months rather than being culled, which could bias seasonal results. In Atlantic Canada, milk production is decreased in some dairy herds in the summer and fall (Dohoo and Ruegg, 1993). Under Canadian milk management policy, farms have daily production targets (www.cdc-ccl.gc.ca). Lower production in summer could decrease dilution of MAP within milk samples and lead to reduced culling to meet milk production targets. Because MAP-infectious cows would typically be at higher culling risk (McKenna et al., 2006; Lombard, 2011), this reduced culling could lead to a greater retention of these cows in summer.

Furthermore, increased stress levels due to crowding, increased milk production, calving, changes in feeding practices and herd management, changes in environment (pasture), adverse weather conditions, and body condition might lead to seasonal trends (Jorgensen, 1977; McKenna et al., 2004; Crossley et al., 2005). In addition to seasonal trends for MAP detection by qPCR in milk samples, we noted a trend for higher contamination of solid culture in summer. The literature suggests that culture decontamination failures may be due to diet and farm location and, therefore, are likely to be affected by clustering (Whitlock et al., 1989; Whittington, 2009). Little is known about such possible causal factors for shedding and decontamination failure patterns, particularly for milk. However, this knowledge is important to enhance specific herd Johne's disease control management and diagnostic protocols and warrants further research. In our study, the small number of farms recruited led us to including farm as a fixed predictor in the model to account for possible confounding, but the inclusion of farm did not affect

the point estimates of the predictor for season. Our study supports the use of milk and colostrum testing by qPCR rather than culture-based methods, especially during periods when the cows are under heavy stress.

CONCLUSIONS

Calves have the highest risk of infection with MAP, and therefore key management strategies must minimize their exposure to the organism via colostrum, milk, and feces. In this study, we analyzed milk and colostrum samples collected over a 12-mo period from a group of previously identified MAP-infectious dairy cows. Traditional culture assays can take from less than 2 mo up to 4 mo, depending on culture method, to obtain a result, which can be futile for decreasing calf exposure in the calving pen. In addition, results from samples collected several months before calving are not necessarily indicative of those cows being infectious at calving, and negative fecal assays did not imply negative milk or colostrum assays and vice versa. Therefore, our data indicated that qPCR, which can provide a result within 24 h if required, showed an improved sensitivity compared with culture assays. Furthermore, qPCR could be used to test for fecal shedding before calving, and to test for MAP in colostrum and early-lactation milk before use or before storage. Our study also found higher qPCR sensitivity in summer months and more synchronization between milk and fecal shedding in summer. Patterns of MAP shedding in milk and colostrum can reveal more efficient detection strategies for these samples. Future research should include repeating seasonal assessments on assay sensitivity over a several-year period while monitoring other management and cow-level predictors and using larger sample sizes, particularly for colostrum analyses.

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